Saskatchewan Agriculture, Food and Rural Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020039

DEVELOPMENT OF A RAPID IR SCREENING METHOD FOR DETERMINTATION OF CYANOGENIC GLUCOSIDE CONTENT IN FLAX AND THE IDENTIFICATION OF LOW CG'S FLAX

Funded by: The Agriculture Development Fund

April, 2005
Prepared by: University of Saskatchewan

105-05133/

RAPID IR SCREENING METHOD FOR DETERMINATION OF CYANOGLUCOSIDE CONTENT

Final Report
SDAFRR 20020039-75AD

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December 2004.

Contents

Project Goals and Milestones: 2
Resources used in achieving milestones 3
Introduction 4
Introduction to the Technique 6
Raman Spectroscopy 9
Nuclear Magnetic Resonance – alternative technology 10
Results and Discussion 13
Summary of IR technique 20
Corroborative Studies using ^{1}H and ^{13}C NMR. 21
Analysis of CG using gas chromatography 24
Conclusions: 25

Abstract

This project was undertaken to assess IR spectroscopy as a rapid, cost effective, and reliable screening technique for cyanogenic glucoside content in flax. The results are strongly suggestive that Raman-scattering IR based spectroscopy will be useful in detecting high CG flax lines. Among a consistently prepared sample set IR spectroscopy may reliably rank materials with similar reporter substances. However, IR spectroscopy techniques cannot be deemed quantitative. Challenges were also encountered with signal to noise in the region of interest. However, this factor may be resolved using more intense light, such as the CLS. Improvements in this area will achieve better resolution and more sensitive detection.

Project Goals and Milestones:

This project was undertaken to develop a cost-effective, reliable IR-based screening technique for cyanogenic glucoside content in mutant flax lines. The flax component of livestock feeds is constrained by a requirement to limit exposure to these anti-nutritional compounds. IR technology offers important additional information such as oil content. However, significant technical challenges exist in implementation.

The goal of this project was to assess IR spectroscopy as a rapid, cost-effective, and reliable screening technique for cyanogenic glucoside content in flax.

Resources used in achieving milestones.

The majority of the resources supported personnel. Shawnee Eidt was engaged to prepare the flax extracts and Eddie Bolster to explore the relative benefits of FTIR or FTIR-Raman spectroscopy and Allyson Anne to confirm CG content using NMR and GC. Material used in the project included solvents, disposable laboratory supplies and data storage CD's. Repairs were performed on the FTIR spectrometer at the Physics Department machine shop and an upgrade to the control computer was purchased.

Introduction

The presence of cyanogenic glycosides (CGs) in fodder carries a potential danger because of the production of cyanide (HCN) via hydrolysis. Animals exhibit variable sensitivity to CGs. Intoxication in cattle is accompanied by sign of anorexia, weakness, depression, stupor, circling, excessive salivation and tenesmus. Clinical examination established ruminal stasis, bright red mucous membranes, tachycardia with cardiac dysrhythmia and scant tarry faeces. Animals may become recumbent with generalized fasciculation; death follows within 10h of onset of clinical signs. In these cases, post mortem findings are consistent with cyanide poisoning, whole blood cyanide concentration has been reported at 0.53 mg/L [1]. Given the severe outcome following intoxication it is important to limit access to feeds that contain significant CG concentrations. We have focused our attention on flax seed, it contains many beneficial elements including omega-three fatty acid but has significant CG concentration. Traditional breeding techniques have not yielded low CG varieties, prompting an alternative approach. This project will test the CG content in mutagenized seed lines to isolate those exhibiting low CG concentrations.

Cyanide exerts its toxic effect by forming a complex with ferric ion (Fe+3) of mitochondrial cytochrome oxidase, the enzyme that catalyzes the terminal step in the electron transport chain, thereby preventing use of oxygen by cells. Since cytochrome oxidase occupies a central role in the use of oxygen in all cells, its inhibition leads to the disruption of cellular respiration producing cytotoxic hypoxia. In addition to binding to cytochrome oxidase, cyanide combines with approximately 2% of methemoglobin normally present (Hardy and Boylen, 1983)

There exist at least six commonly occurring CG's in agricultural materials. Surprisingly these are poorly characterized… little information is available on relative toxicity, interactions, or lability. It is generally thought that the toxicity of CG's arises during their metabolism… with the production of cyanide and various cyanates. However, a comprehensive analysis of this effect has not been reported. Here, we accept that the presence of these compounds in flax restricts the use of this otherwise desirable plant product.

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[1] Hardy, H.L. and G.W. Boylen, Jr. 1983. "Cyanogen, hydrocyanic acid and cyanides." In: Encyclopaedia of Occupational Health and Safety, 3rd ed, Vol. 1, L. Parmeggiani, ed. International Labour Office, Geneva, pp. 574-577.

Traditional approaches for qualitative, semi-quantitative or quantitative determinations of CGs include direct methods, extraction and analysis and indirect methods, hydrolysis and quantification of HCN.

A method for the direct estimation of linamarin in beans and cassava flour has been reported. The sample is extracted with a mixture of acetonitrile and water, cleaned using a solid-phase extraction cartridge then trimethylsilated. Linamarin was quantified by FID-gas chromatography and confirmed by GC/MS (detection limits of linamarin were 0.1 – 1 mg/g plant material).

Nuclear magnetic resonance technology is useful to identify functional groups. Here each chemical group is represented by a unique signature. These signatures are resolved along a frequency axis that is specific for the nucleus studies and its chemical environment. While ^{1}H-NMR is most sensitive, other nuclei can also be studied. In the context of this investigation, ^{13}C-isotope of carbon is a promising candidate. First, the cyanide resonance is well separated from other chemical constituents, second, little sample preparation is required. A drawback of this techniques is that it is difficult to automate, and is relatively insensitive , precluding single seed analysis.

Typically indirect methods are based on the quantification of HCN content liberated from the original plant material. A classical test is based on the reaction of alkaline picrate paper with HCN liberated in a closed test tube by enzymatic hydrolysis of plant macerate. Cyanide can be detect ed at 30 – 5 0 m g/g concentration.

Here we investigate the use of infrared spectroscopy as a direct measurement of CGs in flaxseed. Elaborate sample preparation was avoided by using simple extraction procedures or directly in seed macerates. The presence of CG's was confirmed using FT-^{13}C-NMR.

Introduction to the Technique

1. Fourier Transform Infrared Spectroscopy

The infrared spectrometer used was the BIO-RAD FTS-60 FT-IRS shown in the figure below. This is a new generation FT-IR Spectrometer utilizing interferometry technology. The whole system may be divided into three sub-systems: FTS-60 Optical Bench, Win-IR Analysis software and peripheral equipment.

FTS-60 Optical Bench or spectrometer is responsible for obtaining infrared spectrum. The main component of the spectrometer is a high quality Michelson air bearing interferometer. The interferometer contains a fixed mirror, a movable mirror, and a beamsplitter. The beam-splitter transmits half of the incident radiation to the moving mirror and reflects the other half to the fixed mirror. The two beams are reflected back to the beamsplitter by these mirrors, where they recombine. The moving mirror is driven at a constant velocity by a linear motor under computer control to vary the optical path difference and thus to produce an interferogram. This interferogram is transported via PC-Bench interface to the computer where it is converted to a single-beam spectrum in order to complete various analysis under Win-IR environment. The functional relationship of these components is outlined in Figure 2.

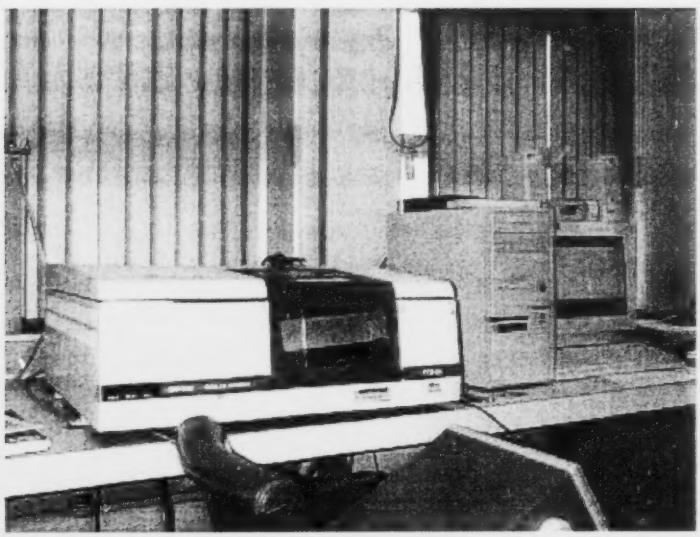

Figure 1. The FTS 60 spectrometer used for the Fourier Transform Infrared Spectroscopy measurements

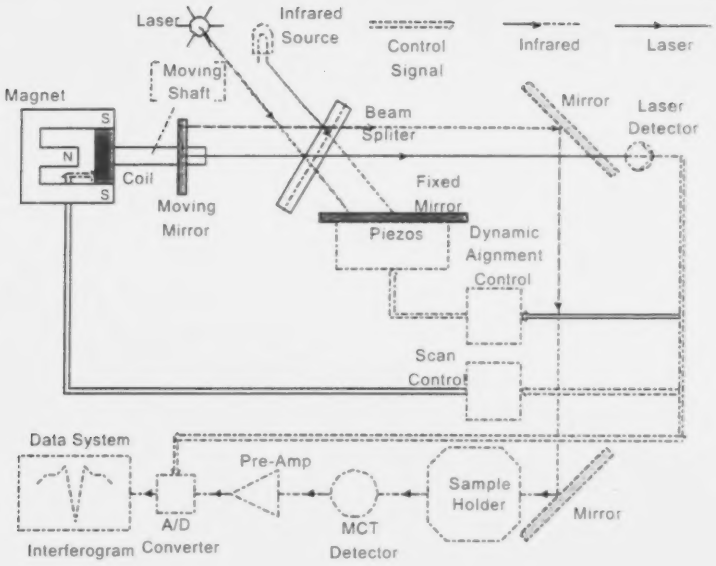

Figure 2. Schematic of FTS-60 Optical Bench

Two data display options are available. A transmission spectrum (T) is produced by dividing the sample single-beam spectrum by the background spectrum.

$$T\% = \frac{\text{Sample Single - Beam Spectrum}}{\text{Background Single - Beam Spectrum}} \times 100 \qquad (2.37)$$

An absorbance spectrum (A) is produced by computing the base 10 logarithm of the reciprocal of the transmittance spectrum.

$$A = \log_{10}\left(\frac{1}{T}\right) \qquad (2.38)$$

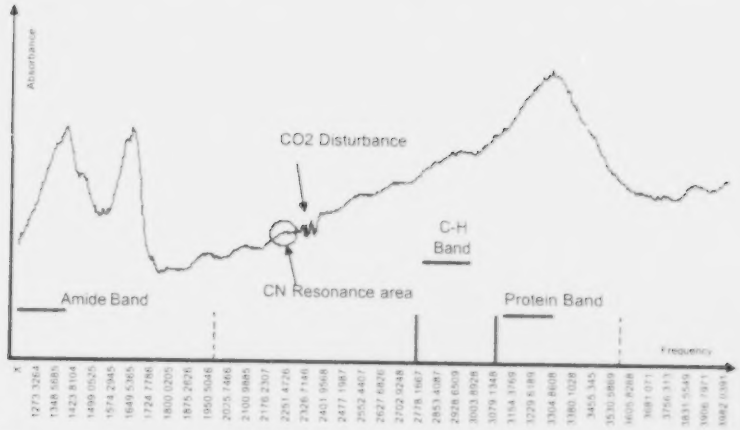

Figure 3. Typical IR absorbance spectrum obtained from an extract.

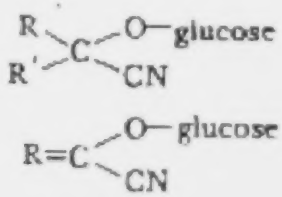

Figure 4. Linamarin, the reference cyanoglucoside used in this study.

Figure 5. FTIR of cyanoglucoside standard linamarin

Raman Spectroscopy

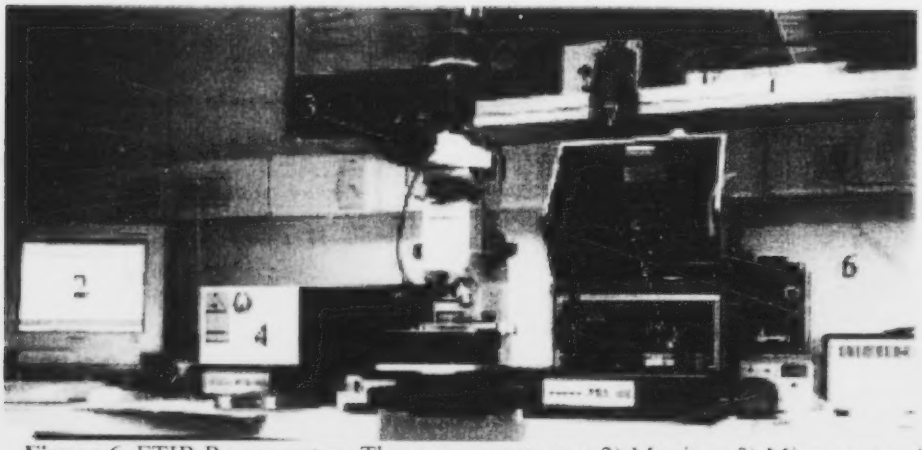

Figure 6. FTIR Raman setup. The components are: 2) Monitor; 3) Microscope; 4) laser source; 5) laser probe; 6) power supply; 7)CCD detector

Principles: When radiation passes through a transparent medium, the species present scatter a fraction of the beam in all directions. A small fraction of the radiation scattered by certain molecules differ from that of the incident beam; furthermore, the shifts in wavelength depend upon the chemical structure of the molecules responsible for scattering. The theory of Raman scattering shows that the phenomenon results from the same type of quantized vibrational changes that are associated with infrared (IR) absorption. Thus, the difference in wavelength between the incident and scattered radiation corresponds to wavelengths in the mid-infrared region. Indeed, Raman scattering spectrum and infrared spectrum for a given species often resemble one another quite closely. There are, however, enough differences between the kinds of groups that are infrared active and those that are Raman active to make the techniques complementary rather than competitive.

For some problems the infrared method is the superior tool, for others, the Raman procedure offers more useful spectra. An important advantage in these experiments of Raman spectra over infrared lies in the fact that water does not cause interference; indeed, Raman spectra can be obtained from aqueous solutions. In addition, glass or

Raman spectroscopy.

Raman light scattering in the IR range offered an attractive alternative. We used the Biorad Raman microscope (Figure 6) located in the Structural Sciences Centre at the University of Saskatchewan. Sample preparation was simplified. Seeds were ground in warm hexane, the hexane decanted and the residue spread thinly on a microscope slide for analysis. Raman spectra are sensitive to the frequency dependent response factor of the equipment. In figure 7 the process of instrument correction is illustrated. Each data spectrum was divided by the corresponding correction spectrum to generate a primary data set.

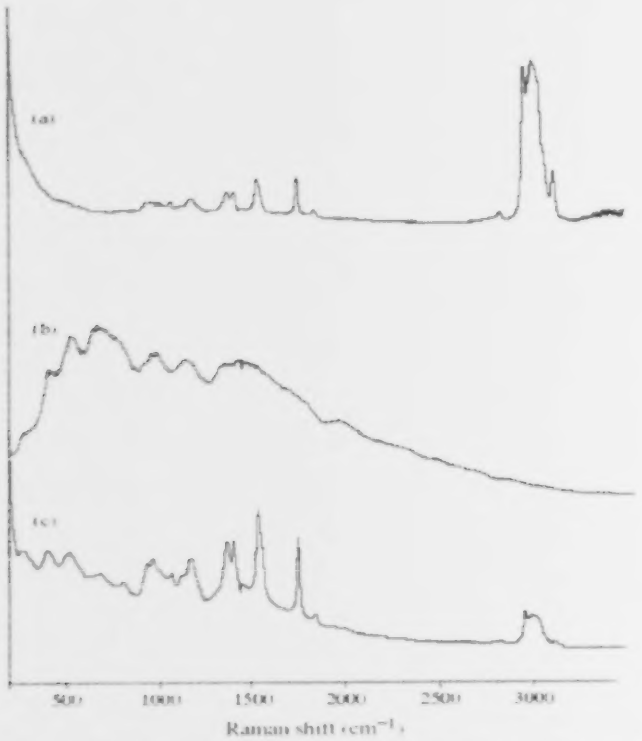

Figure 7. Raman response correction curve. Each spectrum was corrected for frequency dependent differential response. The processed data shown contain data corrected for differential response and background scattering. A) primary data file of a plant macerate. B) Response correction curve; C) derived spectrum, without background correction.

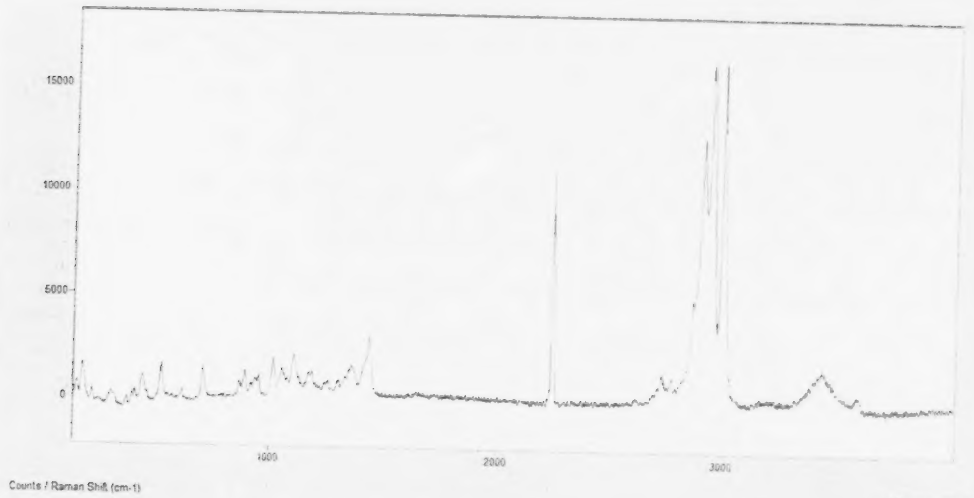

Figure 8. Raman Spectrum of linamarin. Note strong CN resonance at 2236 cm^{-1}.

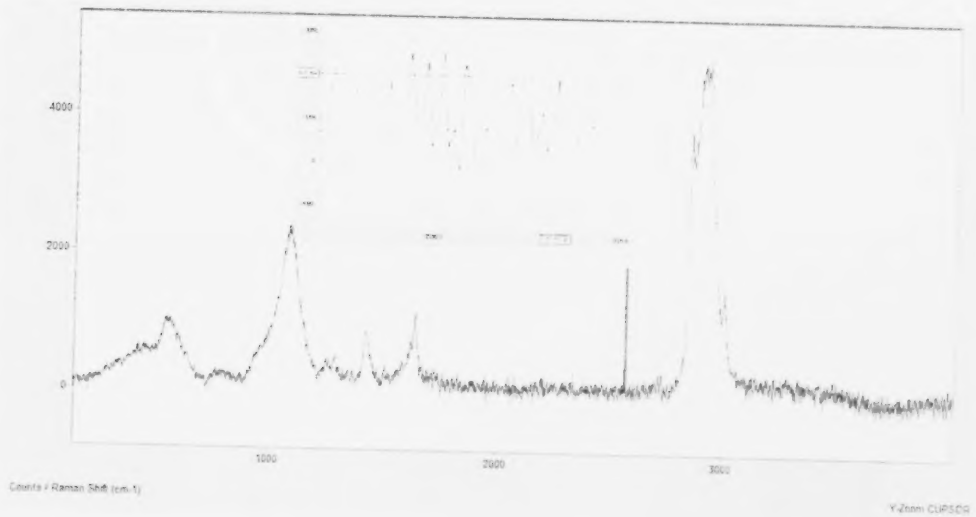

Figure 9. FT-IR Raman spectrum of Valour. Insert shows magnification of the 2220-2240 cm^{-1} range. Vertical line at 2236, the cyanide-stretch Raman shift. Resonances were barely detectable in most cases.

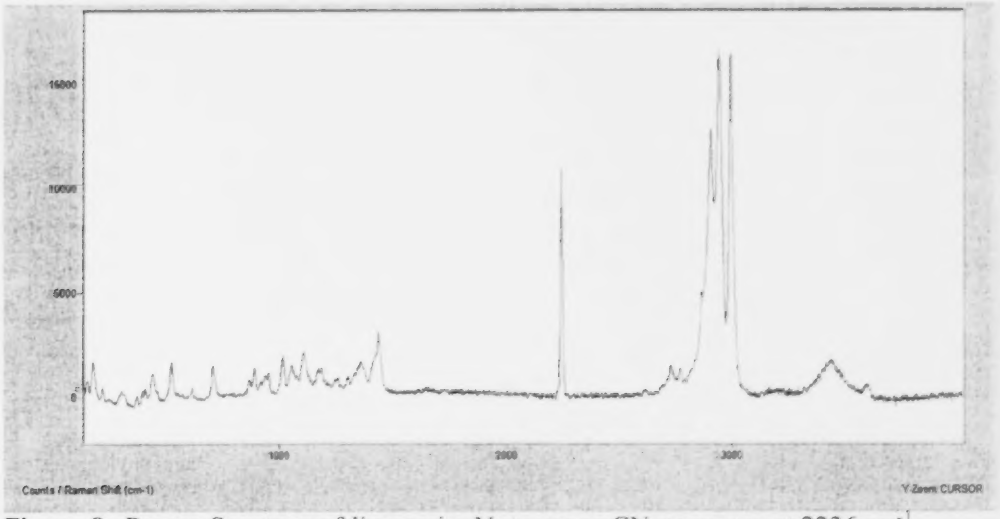

Figure 8. Raman Spectrum of linamarin. Note strong CN resonance at 2236 cm^{-1}.

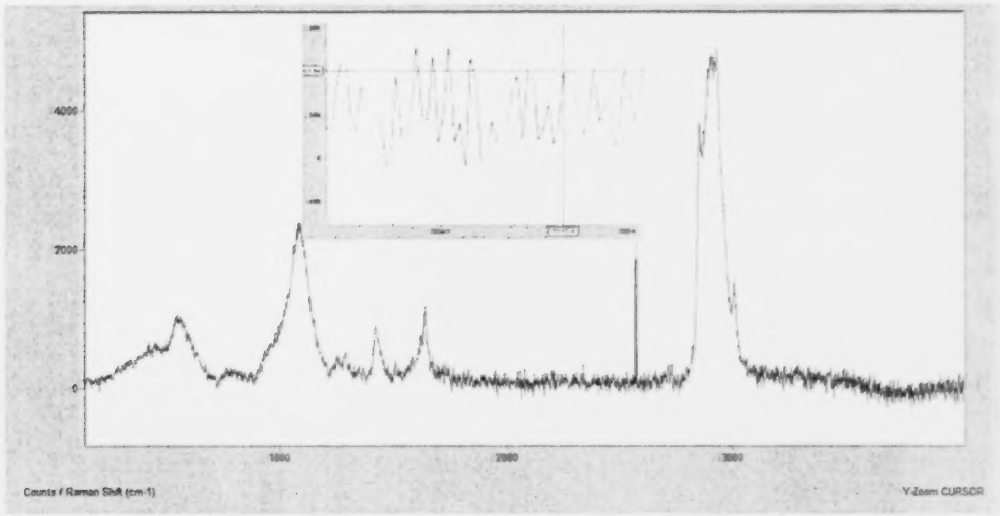

Figure 9. FT-IR Raman spectrum of Valour. Insert shows magnification of the 2220-2240 cm^{-1} range. Vertical line at 2236, the cyanide-stretch Raman shift. Resonances were barely detectable in most cases.

quartz cells can be employed, thus avoiding the inconvenience of working with water sensitive sodium chloride or other atmospherically unstable windows. Thus aqueous solutions can be studied by Raman spectroscopy but not by IR. Raman spectroscopy is subject to interference by fluorescence or impurities in the sample. In brief, a vibrational mode is IR-active when there is a change in the molecular dipole moment during the vibration; a vibrational mode is Raman active when there is a change in polarization during the vibration. Qualitatively, asymmetric vibrational modes and vibrations due to polar groups are more likely to exhibit prominent IR absorption, while a symmetric vibrational mode is often strong in Raman. Molecules with a center of symmetry have no fundamental lines in common in the IR and Raman spectra.

Raman spectra are obtained by irradiating a sample with a powerful laser source of visible or infrared monochromatic radiation. During irradiation, the spectrum of the scattered radiation is measured at some angle (usually 90°) with a suitable spectrometer. At the very most, the intensities of Raman lines are 0.001% of the intensity of the source; as a consequence, their detection and measurement are difficult. This renders the synchrotron, with is extremely bright source, very attractive for detecting low abundance molecules. An exception to this is resonance Raman lines that are considerably more intense.

Raman peaks are found in identical shift patterns, either side of the on-resonance (Rayleigh) peak. The magnitude of Raman shifts is independent of the wavelength of excitation. The appearance of Raman spectral lines at lower energies relative to the excitation energy (longer wavelengths) resembles the Stokes shifts found in a fluorescence experiment (fluorescence absorption and re-emission). Shifts toward higher energies are termed anti-Stokes and are less intense than the corresponding Stokes lines. It is noteworthy that fluorescence may interfere seriously with the observation of Stokes shifts but not with that of anti-Stokes shifts. With fluorescing samples, anti-Stokes signals may therefore be more useful, despite their lower intensities. It is anticipated that the flax samples will exhibit significant fluorescence due to the complex nature of the preparation.

Nuclear Magnetic Resonance – alternative technology

As an alternative to FT-IR measurements the utility of using nuclear spectroscopic techniques was investigated. Nuclear magnetic resonance (NMR) spectroscopy while relatively insensitive is highly specific for functional group identification. Nuclei possess

an intrinsic property termed "spin" that derives its behavior from the constituent sub-atomic particles. For nuclei with an odd atomic number, this property cause degeneracy when they are placed in a magnetic field. As a result the cohort of spins populates distinct energy states where the energy difference is determined by the nucleus (?) and by the strength of the field (B_0).

$$\Delta E = \gamma \ B_0$$

Biologically relevant nuclei like Hydrogen and Carbon (^{13}C a naturally occurring isotope of ^{12}C is the relevant species) populate a low and a high energy state according to a Boltzman's distribution:

$$N_{lower} / N_{higher} = \exp(-\Delta E / kT)$$

where k is the Boltzman's constant and T the absolute temperature. This population excess ($\leq$3.6 e-5 spins at room temperature) is sufficient to produce a measurable signal. The frequency at which this signal appears is primarily a function of the nucleus but is modulated by the presence of proximal nuclei. This chemical based shift gives rise to a unique signal for many functional groups.

The cyanide functional group is relatively electronegative and is present as a distinct

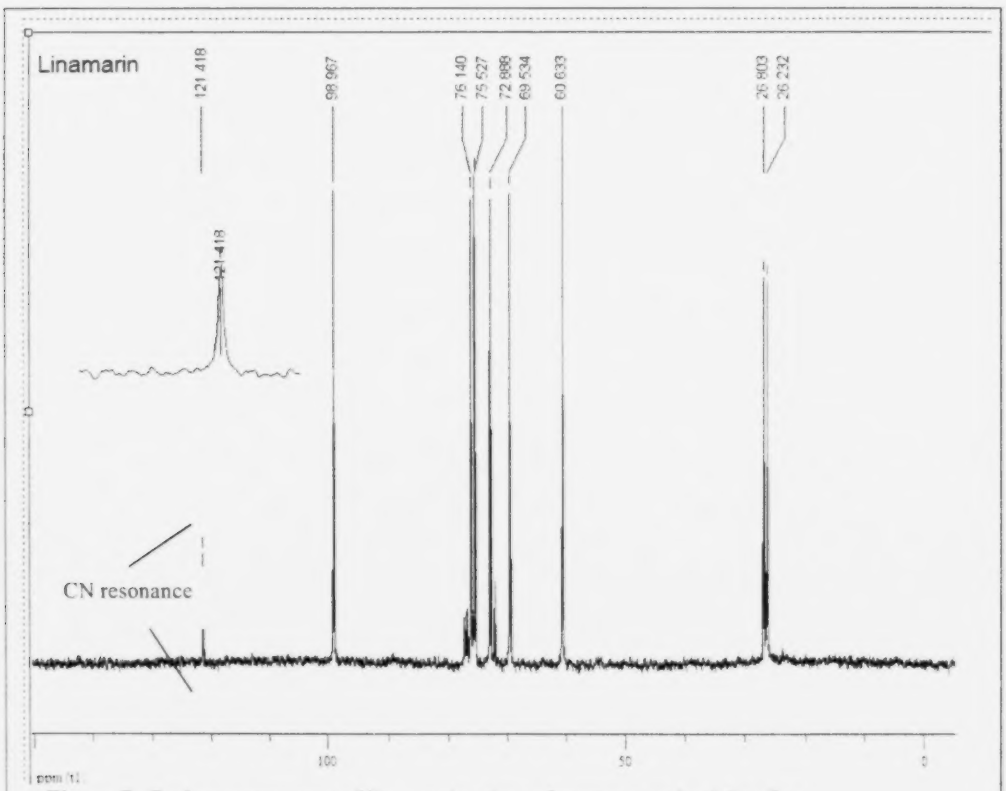

Figure 7. Carbon spectrum of linamarin, the reference standard for flax cyanoglucosides. The insert is an expansion of the resonance at 121 PPM.

signal in ^{13}C-spectra (see Figure 7 below). While highly specific, this technique is relatively insensitive. A more sensitive NMR protocol uses the ^{1}H- nucleus. The presence of a cyanide group alters the chemical shift of neighboring hydrogen atoms. This technique relies on the absence of confounding signals. In Figure 8 below the ^{1}H-spectrum of linamarin, our reference standard is shown. Each resonance is identified and the CN induced chemical shift highlighted.

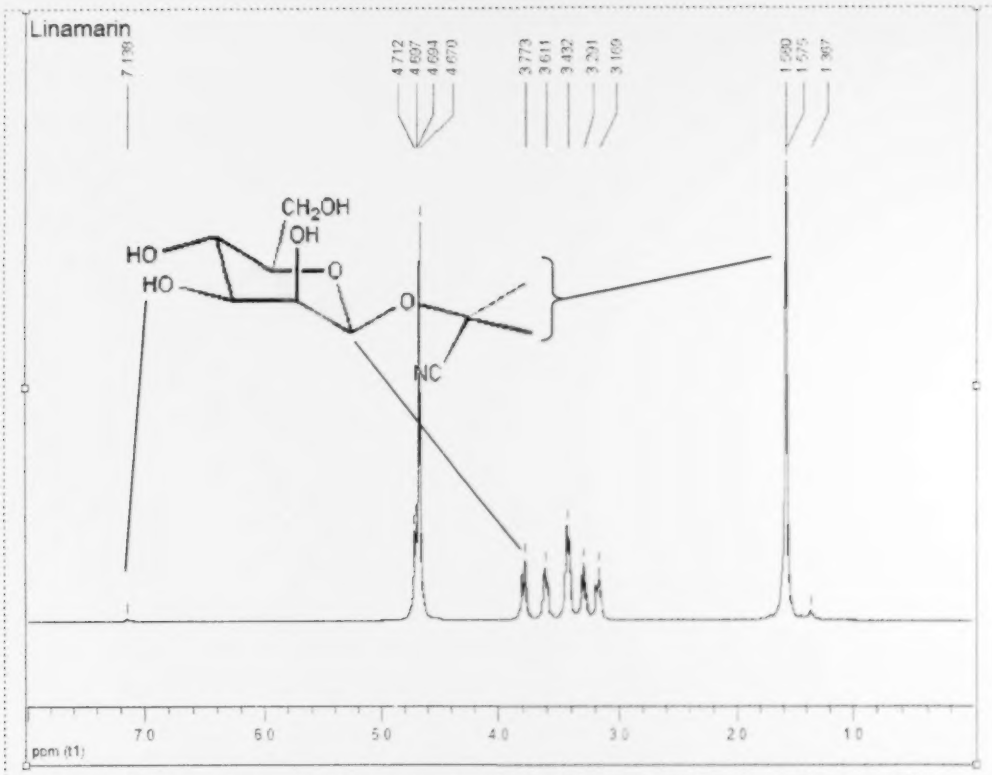

Figure 8. H-spectrum for linamarin. The insert shows the chemical structure of the reference molecule and the arrows identify the corresponding resonance. The doublet at 1.575- 1.580 exhibits 2.5 Hz weak coupling to the CN residue.

The combination of ^{1}H and ^{13}C spectroscopy was used to confirm the presence of CN in the test lines.

Results and Discussion

In this section we report on our efforts to use conventional Infrared Spectroscopy to detect the presence of the cyanide functional group (-C=N) in flax seed extracts. Two technologies were employed: Fourier Transform IR (FTIR) and Raman Scattering IR. (RSIR)

1. FTIRS. We needed to determine how much material would be required to obtain spectra with adequate signal to noise. Typically, thin films were formed by placing a droplet of extract, in this case an alcohol extract, on a NaCl window and evaporating it to residue. The film thickness was increased by repeating the process thereby building successive layers. However, there is a tradeoff between increased functional group concentration and the relative opacity of the film to IR

radiation. Using scattered radiation does not significantly improve the situation due to internal absorption of the scattered radiation. As a result, the experimental procedure was optimized empirically.

a. Alcohol extracts from 0.25 g of seed were combined and evaporated to dryness; thirty two scan averages were collected to produce a spectrum of the reference breeding line "Valour". All experimental lines were sampled similarly.

b. The spectra contained features attributable to the complex carbohydrates, and residual protein. The putatively diagnostic cyanide region was very low in amplitude.

c. Clearly, as anticipated, background fluorescence represented a large problem. The receiver gain for the area of interest (2220 – 2240 cm^{-1}) was limited by the large background fluorescence and scattered radiation from the sample. We considered two approaches to resolving this problem: first, to develop an extraction protocol that enriched the analyte in CG's. This approach would necessitate developing an internal standard to track extraction losses. Considering the theme of the project was to employ relatively simple sample handling and more sophisticated instrumentation, a second approach was adopted.

Figure 9. Typical FTIR spectrum of Flax seed extract. **Above.** Control sample "Valour". **Below**, experimental line "E523".

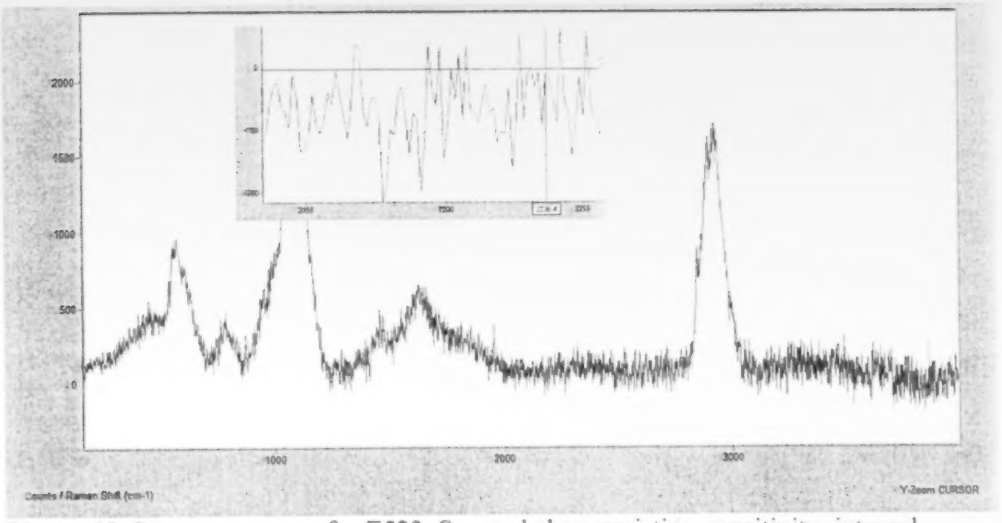

Figure 10. Raman spectrum for E523. Spectral characteristics, sensitivity, internal reference. Insert shows magnification of the 2220- 2240 cm^{-1} range. Vertical line at 2236 is the location for the cyanide-stretch Raman shift. Resonances were barely detectable in most cases.

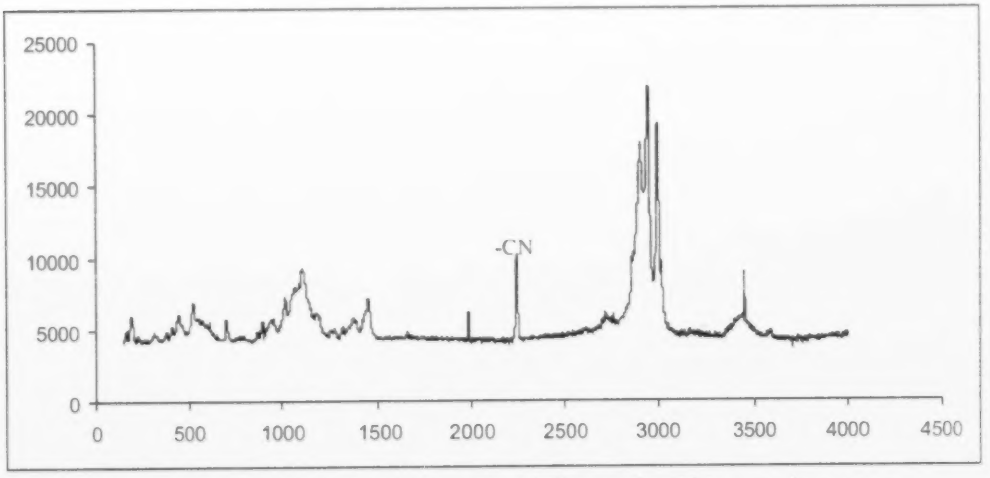

Figure 11. Raman spectrum for E523 doped with linamarin. This experiment was undertaken to determine the detection limit of the bench top spectrometers for CGs *in situ.*

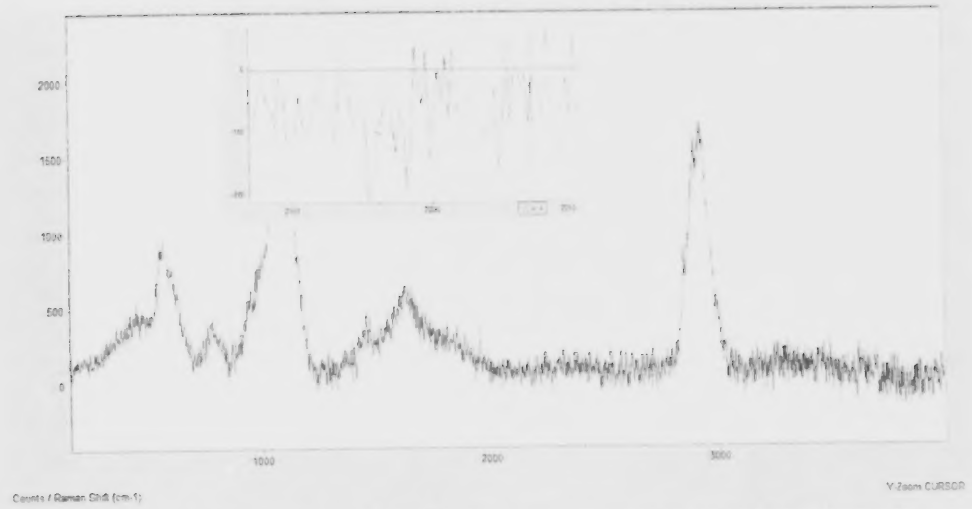

Figure 10. Raman spectrum for E523. Spectral characteristics, sensitivity, internal reference. Insert shows magnification of the 2220- 2240 cm^{-1} range. Vertical line at 2236 is the location for the cyanide-stretch Raman shift. Resonances were barely detectable in most cases.

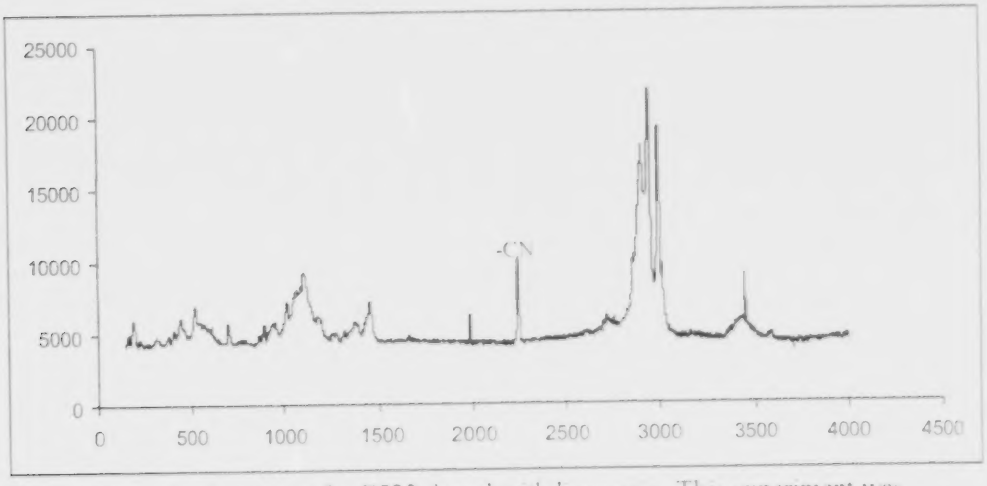

Figure 11. Raman spectrum for E523 doped with linamarin. This experiment was undertaken to determine the detection limit of the bench top spectrometers for CGs *in situ*.

The concentration of CG's in the provided flax lines was unknown. In order to determine a convenient detection limit for CGs in flax, 250 mg of flax seed were ground in warm hexane containing decreasing concentrations of linamarin ranging from 5% to 0.3%. Extrapolating from these values we determined the limit of detection to be 0.26%.

In the following tables the presence of CGs is reported if detected. Relative values 0.26% are essentially not detected. Several lines exhibited CGs in significantly higher ratios that the "control" material, Valour. These lines were created using ethylmethyl sulfonate to non-specifically mutagenize the seed lines. While in many cases this may have generated CG deficient lines, 201 appeared to have a very high CG concentration. These results must be considered preliminary as an exhaustive analysis of error and signal noise has not been performed. However, the approach would seem to be promising in that a range of values were obtained for the twenty-three seed lines analyzed.

Table 1. Analysis of twenty-three flax lines for Cyanoglucosides using FTIR Raman spectroscopy.

Flax line	Peak Intensity Absolute	Peak Intensity Relative
Valour	4121	0.81
201	10462	10.2
202	4516	0.90
203	6373	0.91
214	353	0.35
221	138	0.21
222	48	0.10
223	150	0.25
224	222	0.38
225	3438	0.82
231	102	0.16
232	183	0.22
233	64	0.08
234	62	0.08
235	288	0.41
236	90	0.16
237	127	0.21
241	261	0.57
242	288	0.54
243	229	0.23
477	6892	0.92
1504	5161	0.91
1905	198	0.33
523	6464	0.95

Summary of IR technique

Here we report our preliminary investigations on the suitability of bench-top FTIR spectroscopy for the detection of cyanoglucosides in flax seeds. Our major findings to date are: 1. FTIR spectroscopy suffers from excessive background due to non-specific fluorescence. It was deemed not suitable without: a. elimination of the fluorescence possibly using a Kerr shutter. b. Detector limits preclude post-processing approaches to eliminating fluorescence. 2. FIIR Raman spectroscopy offers promise in detecting CGs. Fluorescence problems are less severe and sample preparation is simplified. On the other hand, the technique is less sensitive requiring a bright excitation source. This technique might be adapted to one of the IR beamlines at the CLS.

Corroborative Studies using ^{1}H and ^{13}C NMR.

A subset of the material was subjected to NMR analysis. Ethanolic extracts were prepared from 300mg seed samples. The samples were defatted using a hexane extraction; the residue was then extracted using 80% ethanol. These extracts were dried under air (~ 100mg DW) and taken up in 0.5 mL D_2O. Spectra were obtained under standard conditions. In brief, proton decoupled ^{13}C data were obtained at 125.77 MHz scanning a frequency range of 125KHz. Excitation was achieved using a 90° pulse. 5200 scan averages were collected with 4.2 s relaxation delay into 16k points. The total acquisition time was 6 h. For 1H spectroscopy the relaxation delay was 4 s and the central frequency 500.13 MHz. Eight scan averages were collected with a scan width of 8170 Hz in 32K points. The total acquisition time was 40 s.

Representative spectra for Valour and a test line are provided in Figures 12 and 13. Known functional groups are labeled. For the ^{13}C spectra, the cyanide groups are evident at 103 and 121 PPM. In the ^{1}H spectra the CH_3 doublet occurs at 1.58 PPM

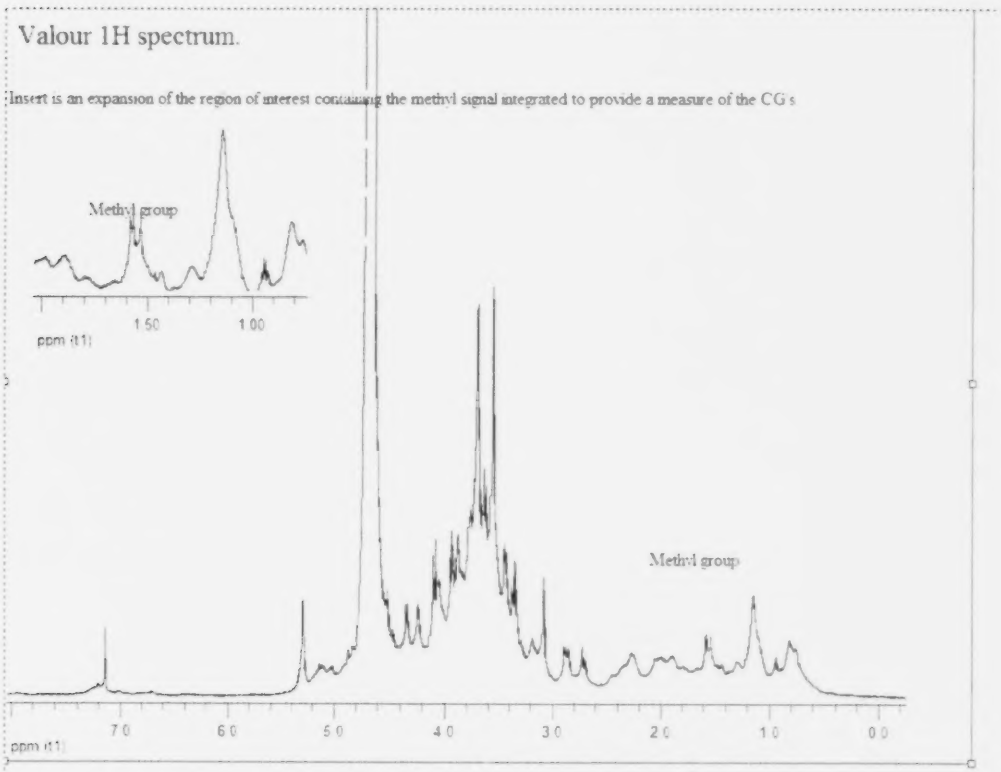

Figure 10. Proton spectrum flax seed extract. The large peak centered at 4.7 PPM is residual water. The small peak at 7.1 PPM is attributed to the sugar hydroxyl groups. The methyl groups of interest resonate at 1.57 PPM.

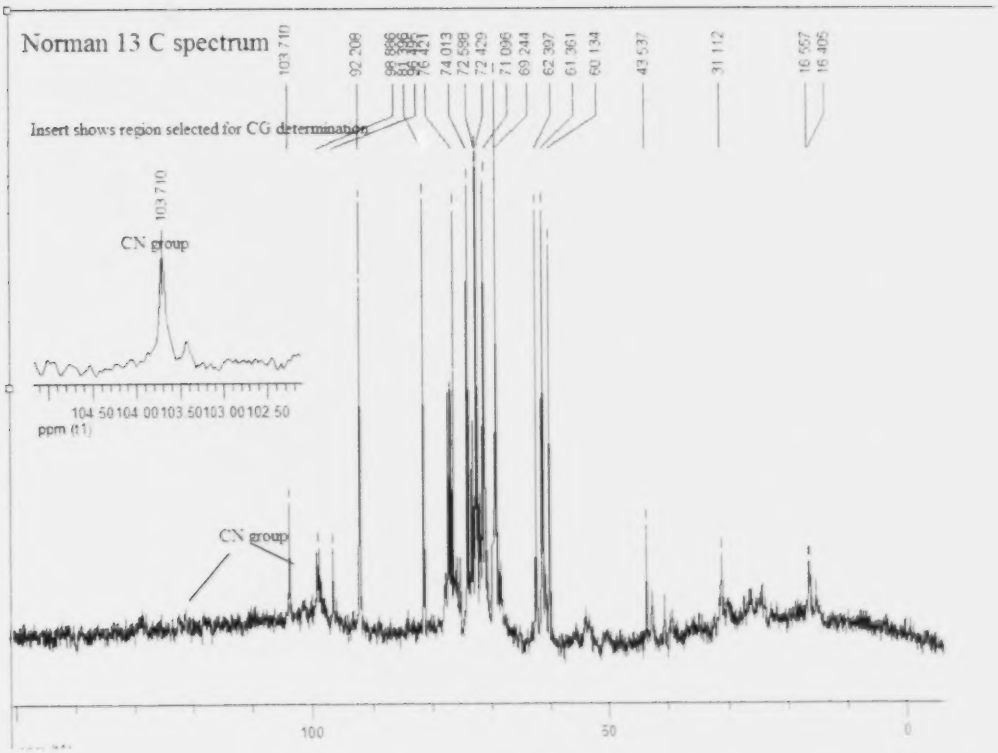

Figure 11. ^{13}C spectrum of variety Norman. The cyanide resonance is featured near 103 PPM.

Table 2. CG content (mg/g seed) in selected flax lines as determined by ^{1}H and ^{13}C NMR spectroscopy.

Line	^{1}H (mg/g)	^{13}C (mg/g)
Valour	0.71a	0.49eg
Norman	1.12b	0.74a
F1504	0.96ab	0.87a
IN732	1.30bc	0.83a
E477	0.85a	0.18f
E523	0.37e	0.67ag

Values in a column or row followed by a different letter are significantly different at the 95% confidence level.

Analysis of CG using gas chromatography.

The extracts were analyzed by gas chromatography after silylation. The method followed established protocols (Adams et al., Biocem System Ecol 18(2/3):107). In brief, alcoholic extracts from approximately 300 mg of seed were evaporated to dryness, then re-dissolved in dimethylformamide (200uL) doped with 50ug of arabinose as an internal standard. This material was derivitized using using BSTFA and 1% TMCS.. The silylated extracts were analyzed by GLC on a DB1701 capillary column using flame ionization detection. As these materials were relatively "dirty", the injector chamber was packed with glass wool to adsorb non-volatile components.

Table 3 CG content in selected flax lines as determined by gas chromatography. Concentrations are expressed as mg CG per mg seed.

Flax line	CN (mg/g seed)
Valour	0.4594
201	1.1664
202	0.5035
203	0.7105
214	0.0394
221	0.0154
222	0.0054
223	0.0167
224	0.0248
225	0.3833
231	0.0114
232	0.0204
233	0.0071
234	0.0069
235	0.0321
236	0.0100
237	0.0142
241	0.0291
242	0.0321
243	0.0255
477	0.7684
1504	0.5754
1905	0.0221
523	0.7207

The results of this analysis are summarized in the Table 3 above. While the ranking of the seed lines did not correspond exactly with that provided by FTIR spectroscopic analysis, there was general agreement between the methods in identifying high and low CG containing lines.
These results confirm our hypothesis that IR based measurements will be useful in identifying high CG flax lines in a screening protocol.

Table 4. Comparison of CG concentration as determined by Raman IR scattering, proton spectroscopy, carbon spectroscopy and gas chromatography

	IR (%)	^{1}H (mg/g)	^{13}C (mg/g)	GC (mg/g)
Valour	0.81	0.71	0.49	0.46
F1504	0.91	0.96	0.87	0.58
E477	0.92	0.85	0.17	0.76
E523	0.95	0.37	0.67	0.72

In Table 4 we compare the CG ranking values obtained from different techniques. Each technique assesses different features of the cyanoglucosides. For example, the IR and ^{13}C techniques directly measure the presence of –CN in the sample material. The lines examined all fell into the "high" category according to the IR measurements. However, ^{13}C spectroscopy may have placed E477 in a low CG category.

In contrast, ^{1}H and GC methods infer the presence of CG's based on reference information. If we arbitrarily take 0.2 mg/g as a lower cutoff and 1.0 mg/g as an upper limit, these data suggest that all tested lines contain similar levels of CG's. This is in agreement with the IR findings. The cutoff level for these classifications may require further refinement if the method is to be scaled from the experimental to the industrial setting.

Conclusions:

This project was undertaken to determine if IR spectroscopy, either in traditional absorption mode or in a Raman scatter configuration, could screen flax seeds for cyanoglucoside content. Our findings suggest that this may not be possible on intact seeds, but may be possible with simple macerates. IR spectroscopy is not an easily quantifiable methodology. However, among a consistently prepared sample set, it may reliably rank materials with similar reporter substances. This was evidenced here by comparing the results of Raman and GC analyses of the seed lots. The principle advantages of the

approach include ease of sample preparation, amenability to automation, rapid analysis time.

A second method, NMR spectroscopy was also evaluated using a small number of seed lines. Proton spectroscopy is intrinsically quantitative but, in the case of a functional group without hydrogen (cyanide), relies on indirect measurements. Carbon based spectroscopy requires additional steps for measurement, for example the any attached hydrogen moieties are normally "decoupled" from the carbon spectra. This introduces additional variability in the spectra as decoupling tends to alter the peak height. While these techniques did address the primary question of detection, the results were not consistent with the findings from GC. Thus, the recommendation fo

The results are strongly suggestive that Raman-scattering IR based spectroscopy will be useful in detecting high GC flax lines. While the technique cannot be deemed quantitative, screening may be achieved using standardized protocols. We encountered challenges with signal to noise in the region of interest. This factor may be resolved using more intense light, such as that soon to be available at the CLS. Improvements in this area will achieve better resolution and more sensitive detection.

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